Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Quantitating the GP70 Antigen of Feline Leukemia Virus Veterinary Vaccines

Date: December 22, 1999 Draft--Approved, pending Standard Requirement

Supersedes: Draft dated, April 1, 1994

Number: MVSAM0321.01

Standard Requirement: Draft

Contact Person: Sally Hanna, (515) 663-7337

Approvals:

_____ Date:_____
Linn A. Wilbur, Head/Team Leader
Mammalian Virology Section

_____ Date:_____
Ann L. Wiegers, Quality Assurance Manager

/s/ Linda L. Schlater for Date: 12/22/99
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for Quantitating the GP70 Antigen of Feline Leukemia Virus Veterinary Vaccines

Table of Contents

Supplemental Assay Method for Quantitating the GP70 Antigen of Feline Leukemia Virus Veterinary Vaccines

1. Introduction

1.1 Background

This is a Supplemental Assay Method (SAM) enzyme-linked Immunosorbent Assay (ELISA) titration method for quantitation of the 70,000 Dalton glycoprotein (gp70) antigen of feline leukemia virus (FeLV) vaccines by the relative potency (RP) method. The RP of a Test Serial is determined by comparing the amount of gp70 antigen of a Test Serial to the gp70 antigen content of a Reference Preparation that has been directly or indirectly shown to be protective in a host animal immunogenicity trial.

1.2 Keywords

Feline leukemia, FeLV, *in vitro*, RelPot, ELISA

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator $36^0 \pm 2^0$C, 5% ± 1% CO_2, high humidity,[1] meeting the requirements of the current version of GDOCSOP0004

2.1.2 Microplate reader[2]

2.1.3 Microplate washer[3]

2.1.4 Micropipettors: 200 µl and 500 µl single channel,[4] 50-200 µl x 12 channel,[5] and tips[6]

---

[1] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent
[2] Model MRX, Dynex Technologies, Inc., 14340 Sullyfield Circle, Chantilly, VA 20151 or equivalent
[3] Model EL404, Bio-Tek Instruments, Inc., Highland Park, Box 998, Winooski, VT 05404-0998 or equivalent
[4] Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[5] Cat. No. 77-705-00, Flow Laboratories, 7655 Old Springhouse Road, McLean, VA 22102 or equivalent
[6] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent

2.1.5 RP Calculation Method: Use the current version of *SAM for Evaluation by the Relative Potency Method of In Vitro Enzyme Immunoassays Used In Testing of Veterinary Vaccines (MVSAM0318).*[7]

2.1.6 Current version of the U.S. Department of Agriculture, Center for Veterinary Biologics (CVB) Program's *Relative Potency Calculation Software* (*RelPot*).[7]

2.2 Reagents/supplies

2.2.1 0.01 M Phosphate buffered saline (PBS)

2.2.1.1 1.33 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[8]

2.2.1.2 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)[9]

2.2.1.3 8.5 g sodium chloride (NaCl)[10]

2.2.1.4 Q.S. to 100 ml with distilled water (DW)

2.2.1.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)[11] or 1.0 N hydrochloric acid (HCl).[12]

2.2.1.6 Sterilize by autoclaving at 15 psi, $121^0 \pm 2^0$C for 35 ± 5 min.

2.2.1.7 Store at $4^0 \pm 2^0$C.

2.2.2 0.05 M Carbonate Coating Buffer, pH 9.6

2.2.2.1 0.159 g sodium carbonate (Na_2CO_3)[13]

---

[7] Available on request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010
[8] Cat. No. S 0876, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[9] Cat. No. S 9638, Sigma Chemical Co. or equivalent
[10] Cat. No. S 9625, Sigma Chemical Co. or equivalent
[11] Cat. No. S 925-30, Sigma Chemical Co. or equivalent
[12] Cat. No. 920-1, Sigma Chemical Co. or equivalent
[13] Cat. No. S 1641, Sigma Chemical Co. or equivalent

2.2.2.2 0.293 g sodium bicarbonate ($NaHCO_3$)[14]

2.2.2.3 Q.S. to 100 ml with DW.

2.2.2.4 Adjust pH to 9.6 with 1.0 N HCl.

2.2.2.5 Store at $4^0 \pm 2^0$C; use within 1 wk.

2.2.3 Blotto

2.2.3.1 1.5 g nonfat dry milk powder[15] (Brands and lots may vary in blocking ability. Determine appropriate percentage of dry milk for each batch.)

2.2.3.2 100 ml PBS

2.2.3.3 Add 2 µl of Antifoam A.[16]

2.2.3.4 Store at $4^0 \pm 2^0$C; use within 1 wk.

2.2.4 Diluent Buffer

2.2.4.1 100 ml Blotto

2.2.4.2 1.0 ml Triton X-100[17]

2.2.4.3 Store at $4^0 \pm 2^0$C; use within 1 wk.

2.2.5 Washing Buffer

2.2.5.1 1.0 L PBS

2.2.5.2 1.0 ml Tween-20[18]

2.2.5.3 Store at $4^0 \pm 2^0$C.

---

[14] Cat. No. S 6014, Sigma Chemical Co. or equivalent

[15] *Flavorite*® Instant Nonfat Dry Milk, extra grade, Preferred Products, Inc., 11095 Viking Drive, Eden Prairie, MN 55344 or equivalent

[16] Cat. No. A 5758, Sigma Chemical Co. or equivalent

[17] Cat. No. X-100, Sigma Chemical Co. or equivalent

[18] Cat. No. 170-6531, BioRad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547 or equivalent

2.2.6 Goat Anti-gp70 Polyclonal Antibody[19]

2.2.7 Anti-gp70 monoclonal antibody[20] (MAb)

2.2.8 Goat anti-mouse horseradish peroxidase conjugate[21] (Goat Anti-mouse Conjugate)

2.2.9 (2,2'azino-di-{3 ethyl-benzthiazaline sulfonate 6})(ABTS) peroxidase substrate solution[22] (Substrate Solution)

2.2.9.1 Solution A, ABTS

2.2.9.2 Solution B, Hydrogen Peroxide

2.2.10 gp70 Positive Control[6]

2.2.11 Reference preparation: Each manufacturer provides a Reference Vaccine that has been directly or indirectly shown to be protective in a host animal immunogenicity trial. The reference preparation is the lot number identified in Part V of the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production or special outline. All subsequent serials produced by the manufacturer must have an RP equal to or greater than the RP value contained in the APHIS filed Outline of Production.

2.2.12 Immulon II®, flat bottom, 96-well plate[23]

2.2.13 Flat bottom, 96-well plate[24]

2.2.14 Plate Sealer[25]

---

[19] National Cancer Institute Repository, through Quality BioTech, 1667 Davis St., Camden, NJ 06104. Available upon request from the CVB-L

[20] Hybridoma provided to the CVB-L by Dr. Neils Pedersen, School of Veterinary Medicine, University of California, Davis, CA 95616. Ascites available upon request from the CVB-L

[21] Cat. No. 115-035-062, Jackson ImmunoResearch Laboratories, 872 West Baltimore Pike, West Grove, PA 19390 or equivalent

[22] Product Code 50-62-00, Kirkegaard & Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879-4174 or equivalent

[23] Cat. No. 011-010-3450, Dynex Technologies, Inc.

[24] Cat. No. 3590, Costar Corporation, 1 Alewife, Cambridge, MA 02140 or equivalent

[25] Cat. No. 001-010-3501, Dynex Technologies, Inc. or equivalent

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training and experience in the immunological basis of antigen capture ELISA assays, the principles of Optical Densitometry (OD), and computer software analysis.

3.2 Preparation of equipment/instrumentation

On the day of the reading, the ELISA reader must be turned on at least 30 min prior to determination of the OD. Dual wavelength settings, 405-nm test wavelength measured against a 490-nm reference wavelength, are used. The ELISA reader is zeroed on air prior to use.

3.3 Preparation of reagents/control procedures

3.3.1 Test plate preparation. One to 5 days prior to test initiation, dilute the Goat Anti-gp70 Polyclonal Antibody, per the Center for Veterinary Biologics-Laboratory (CVB-L) Reference and Reagent Sheet supplied with the reagent, in Carbonate Coating Buffer.

3.3.1.1 Pipette 100 µl/well of the Diluted Goat Anti-gp70 Polyclonal Antibody to all wells of a flat bottom, 96-well Immulon II® plate. This becomes the Test Plate.

3.3.1.2 Cover the Test Plate with a Plate Sealer and incubate at 68 ± 52 hr, at 4^0 ± 2^0C.

3.3.2 Diluted gp70 Positive Control preparation. On the day of testing, dilute the gp70 Positive Control, per the CVB-L Reference and Reagent Sheet supplied with the reagent, in Diluent Buffer.

3.3.3 Diluted Anti-gp70 MAb preparation. On the day of testing, dilute the Anti-gp70 MAb, per the CVB-L Reference and Reagent Sheet supplied with the reagent, in Blotto.

3.3.4 Diluted Goat Anti-mouse Conjugate. On the day of testing, dilute the Goat Anti-mouse Conjugate, per previously determined optimal dilution, in PBS. Optimal dilution results in an OD reading between 0.400 and 0.700 when incubated at $36^0 \pm 2^0$C for 20 ± 10 min.

3.3.5 Substrate Solution preparation. On the day of testing, just prior to substrate addition, mix equal volumes of ABTS Solution A and Hydrogen Peroxide Substrate Solution B per the manufacturer's instructions. The resulting Substrate Solution should remain clear and should be at room temperature (RT), $23^0 \pm 2^0$C, at time of use.

3.4 Preparation of the sample

3.4.1 Antigen Extraction (Optional). If the Test Serial contains adjuvant which interferes with antigen detection, the firm may specify the procedure for extraction of the antigen from the adjuvant. If extraction is a necessary step, the extraction procedure will be included in Part V of the APHIS filed Outline of Production. The CVB-L will extract antigen using the firm's protocol. If no protocol is stated in either the APHIS filed Outline of Production or special outline, the test will be conducted at the CVB-L without extraction. If the Reference Preparation is a product reference, both the Reference Preparation and Test Serial must be treated identically. If the Reference Preparation is a purified preparation, the extraction procedure is not required.

3.4.2 All samples must be at RT before testing. The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). Make twofold dilutions of the Reference Preparation and the Test Serial in Diluent Buffer in a flat bottom, 96-well plate which becomes the Transfer Plate.

3.4.3 An initial dilution of the Reference Vaccine and/or Test Serial may be made. This dilution is determined by the firm to assure the 6 dilutions tested encompass the linear portion of the regression curve. The starting dilution for the Reference Vaccine and/or the Test Serial shall be stated in Part V of the APHIS filed Outline of Production or special outline. Unless stated otherwise, the diluent will be Diluent Buffer for the initial dilution.

3.4.3.1 Pipette 150 µl of Diluent Buffer to wells in rows C-G of the Transfer Plate with a 12-channel micropipettor (see **Appendix I**).

3.4.3.2 Pipette 300 µl of the starting dilution of the Reference Preparation to 3 wells in row B of the Transfer Plate (see **Appendix I**).

3.4.3.3 Pipette 300 µl of the starting dilution of the first Test Serial to 3 adjacent wells in row B of the Transfer Plate. An additional Test Serial may be tested into the next 3 adjacent wells.

3.4.3.4 With a 12-channel micropipettor and the appropriate number of tips to correspond to the number of wells used in a row, mix row B (7 ± 2 fills) and transfer 150 µl to row C of the Transfer Plate. Replace tips, mix row C, and transfer 150 µl to row D.

3.4.3.5 Continue as in **Section 3.4.3.4** for the remaining rows D-G of the Transfer Plate, transferring 150 µl from the previous to the next row.

4. Performance of the test

4.1 On the day of testing, remove the Plate Sealer and pipette 200 µl of thoroughly mixed Blotto to each well of the Test Plate already containing Diluted Goat Anti-gp70 Polyclonal Antibody. Do not remove the Diluted Goat Anti-gp70 Polyclonal Antibody prior to adding the Blotto. Reseal plate and incubate at $4^0 \pm 2^0$C for 90 ± 30 min.

4.2 Decant Blotto from the Test Plate. Pipette 200-300 µl of Washing Buffer into each well. Immediately decant Washing Buffer from the plate. Repeat for a total of 3 washes. At no time should wells dry between washes or incubations. An automatic microplate washer may be used.

4.3 Transfer 100 µl/well of each dilution in the Transfer Plate (starting in row G, most dilute, and ending in row B, least dilute) to appropriate rows of the Test Plate (see **Appendix I**). Tips need not be changed between rows when proceeding from most dilute to successively more concentrated dilutions.

4.4 Pipette 100 µl/well of Diluted gp70 Positive Control to wells 11-B, 11-C, and 11-D of the Test Plate.

4.5 Pipette 100 µl/well of Diluent Buffer to wells 11-E, 11-F, and 11-G of the Test Plate to serve as blanks.

4.6 Seal the Test Plate with a Plate Sealer; incubate at $36^0 \pm 2^0$C for 60 ± 10 min.

4.7 Wash the Test Plate as in **Section 4.2**.

4.8 Pipette 100 µl/well of Diluted Anti-gp70 MAb to all wells of the Test Plate.

4.9 Seal the Test Plate with a Plate Sealer; incubate at $36^0 \pm 2^0$C for 60 ± 10 min.

4.10 Wash the Test Plate as in **Section 4.2**.

4.11 Pipette 100 µl/well of Diluted Goat Anti-mouse. Conjugate to all wells of the Test Plate.

4.12 Seal the Test Plate with a Plate Sealer; incubate at $36^0 \pm 2^0$C for 60 ± 10 min.

4.13 Wash the Test Plate as in **Section 4.2**, then wash 2 times with PBS instead of Washing Buffer.

4.14 Pipette 100 µl/well of Substrate Solution to all wells of the Test Plate.

4.15 Incubate the Test Plate at RT for 20 ± 10 min.

4.16 Read the Test Plate at 405-nm test wavelength against a 490-nm reference wavelength, when the Positive Control wells (11-B, C, D) read between 0.400 and 0.700 OD after the average blank reading is subtracted.

4.17 The arithmetic mean of the blank wells is determined; this becomes the average blank reading. The average blank reading is subtracted from all readings before analysis of the data by *RelPot.*

4.18 Evaluate results using *RelPot*.

5. Interpretation of the test results

5.1 If the corrected OD of the Positive Control is not within the 0.400-0.700 OD range, the test is considered a **NO-TEST** and can be repeated without prejudice.

5.2 All validity criteria in the current version of MVSAM0318 must be met for a valid test. An invalid test may be repeated. Testing may be repeated for **EQUIVOCAL** tests as defined in 9 CFR, Part 113.8(c)(4).

5.3 For a given Test Serial to be **SATISFACTORY**, the RP value of at least 1 valid RP from the group of the highest scoring valid RP values has to be greater than or equal to the RP contained in an APHIS filed Outline of Production.

5.4 For a Test Serial less than the RP contained in an APHIS filed Outline of Production, the test may be repeated if the test meets 9 CFR, Part 113.8(c)(5) criteria.

6. Report of test results

Results are reported as the RP for the Test Serial. The highest RP of the top scores shall be considered the RP value for reporting purposes.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.8, U.S. Government Printing Office, Washington, DC, 1999.

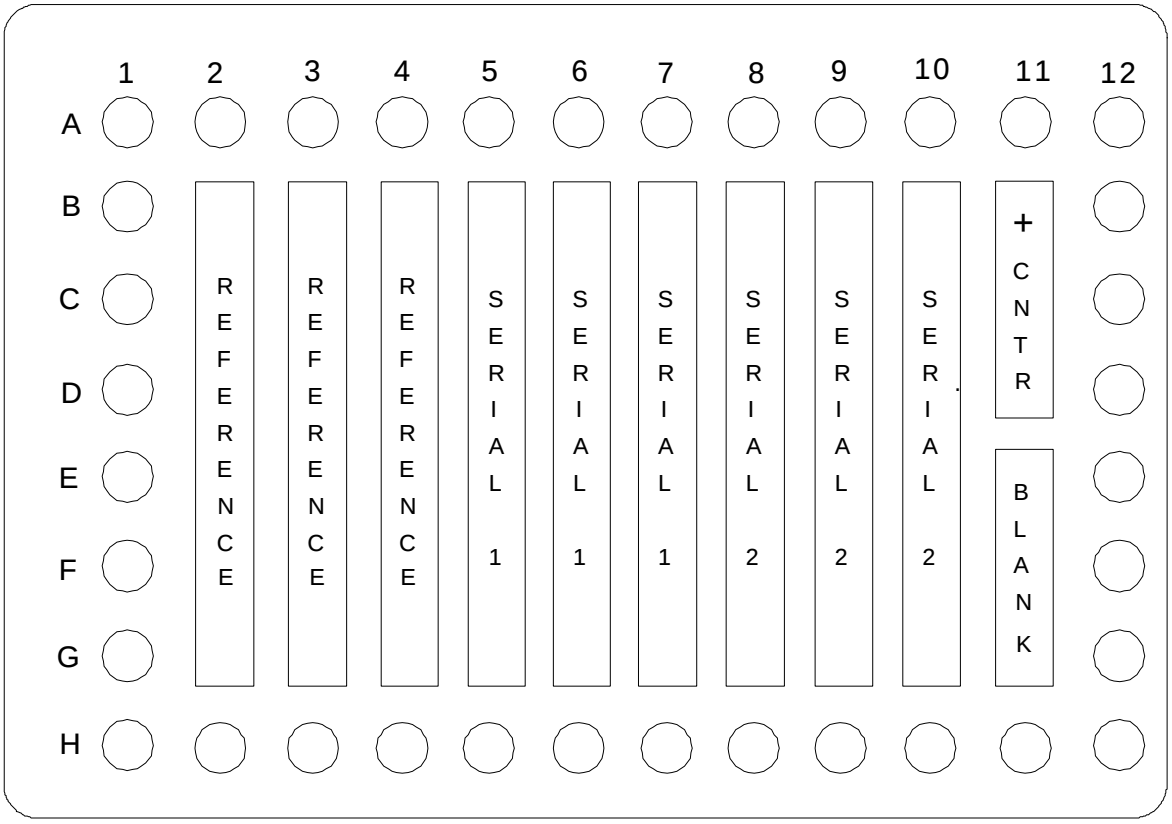
7.2 Shibley GP, Tanner JE, Hanna SA, United States Department of Agriculture licensing requirements for feline leukemia virus vaccines. *JAVMA*, (1991), Vol 199, No. 10, 1402-1406.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous draft protocol.

Supplemental Assay Method for Quantitating the GP70 Antigen of Feline Leukemia Virus Veterinary Vaccines

9. Appendix I: Transfer and Test Plate Format



Locations for dilution plate:

Row B, columns 2, 3, and 4: lowest dilution of Reference Vaccine
Row B, columns 5, 6, and 7: lowest dilution of Test Serial 1
Row B, columns 8, 9, and 10: lowest dilution of Test Serial 2

Locations for test plate:

Rows B through G, columns 2, 3, and 4: increasing dilution of the Reference Vaccine
Rows B through G, columns 5, 6, and 7: increasing dilution of Test Serial 1
Rows B through G, columns 8, 9, and 10: increasing dilution of Test Serial 2
B-11, C-11, and D-11: gp70 Positive Control
E-11, F-11, and G-11: Blank